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Nucleotide sequence coding for an outer membrane protein from Neisseria meningitidis and use of said protein in vaccine preparations

Abstract:

The present invention is concerned with a method for the isolation of a nucleotide sequence which codes for a protein having a molecular weight of about 64 000 daltons, which is located on the outer membrane of N. meningitidis, as well as with the recombinant DNA obtained therefrom, which is used for the transformation of a host microorganism. The technical object pursued with the invention is the identification of a nucleotide sequence coding for a highly conserved and common protein for the majority of pathogenic Neisseria strains, the production of this protein with a high level of purity and in commercially useful amounts using the recombinant way, so that it can be used in diagnostic methods and vaccine preparations with a broad immunoprotection spectrum.

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# (54) Nucleotide sequence coding for an outer membrane protein from Neisseria meningitidis and use

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**of said protein in vaccine preparations.**

(57) The present invention is concerned with a method for the isolation of a nucleotide sequence which codes for a protein having a molecular weight of about 64 000 daltons, which is located on the outer membrane of N. meningitidis, as well as with the recombinant DNA obtained therefrom, which is used for the transformation of a host microorganism. The technical object pursued with the invention is the identification of a nucleotide sequence coding for a highly conserved and common protein for the majority of pathogenic Neisseria strains, the production of this protein with a high level of purity and in commercially useful amounts using the recombinant way, so that it can be used in diagnostic methods and vaccine preparations with a broad immunoprotection spectrum.

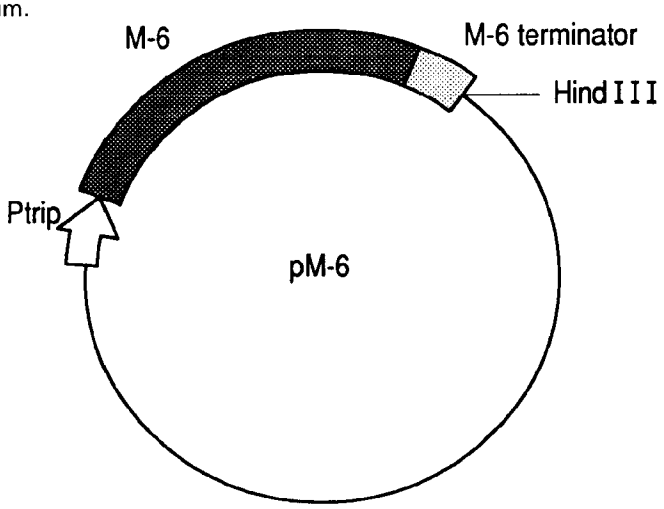



FIG. 1

The present invention is in the field of Genetic Engineering and Biotechnology. More in particular, the invention is related to a nucleotide sequence obtained from the pathogenic bacterium Neisseria meningitidis, which nucleotide sequence codes for a protein belonging to the outer membrane of said bacterium. Said protein is cloned and expressed in the host Escherichia coli. The characteristics of this protein as well as its capacity to induce immunologically active antibodies (bactericidal antibodies) in its natural host, allow its use in vaccine preparations against pathogenic strains of this microorganism.

The gram-negative bacterium N. meningitidis is responsable for one of every three cases of bacterial meningitidis in the world. It was described for the first time by Anton Weichselbaum in 1887 (I. DeVoe, 1982, Microbiol. Revs. 46: 162-190), and man (i.e. human beings) is its only natural host up to date.

In the first half of this century some essential aspects were found in relation to the metabolism and serological differentiation of this microorganism. The first unsuccessful attempts to obtain vaccine preparations were based on its capsular polysaccharide (E. Kabat et al., 1945, J. Exp. Med. 80: 299-307). According to the chemical composition of this capsular polysaccharide, the bacterium N. meningitidis is serogrouped in A, B, C, 29-E, H, I, K, L, W-135, X, Y or Z, and the major percentage of illness is caused by A, C, Y, W-135 and B. Non-encapsulated strains are not associated with the invasive disease.

Using different methods of purification of these polysaccharides (E. Gotschlich et al., 1969, J. Exp. Med. 129: 1349-1365) the four first polysaccharides (PS) showed to be good immunogens and inducers of bactericidal antibodies in humans (E. Gotschlich et al., 1969, J. Exp. Med. 129: 1367-1384). The presence of this kind of antibodies has been correlated previously with non-susceptibility to the infection (I. Goldschneider et al., 1969, J. Exp. Med. 129: 1307-1326). As of today mono-, bi- or tetravalent vaccines have been well studied for serotypes A, C and W-135 (F. Ambrosch et al., 1983, Bulletin of the WHO 61: 317-323; I. Vodopija et al., 1983, Infect. Immunol. 42: 599-604; M. Cadoz et al., 1985, Vaccine 3: 340-342; H. Peltola et al., 1985, Pediatrics 76: 91-96).

These vaccines have been licensed for their use in humans in different countries (Centers for Disease Control, 1985, Morbid. Mortal. Weekly Report 34: 255-259) and some of them are commercially available from different firms and producers (Connaught Laboratories, USA; Smith Kline-RIT, Belgium; Institute Merieux, France; Behringwerke Aktiengesellschaft, Germany; Istituto Sieroterapico e Vaccino genea Toscano "Sclavo", Italy; Swiss Serum and Vaccine Institute, Berne, Switzerland; among others).

However, the conventional vaccine against N. meningitidis serogroup C does not induce sufficient levels of bactericidal antibodies in children under 2 years old, which are the principal victims of this disease. It has been demonstrated that the titer of specific antibodies against N. meningitidis in children under four years of age, after three years of vaccination, is similar in vaccinated and in non-vaccinated ones (H. Kayhty et al., 1980, J. of Infect. Dis. 142: 861-868). Also, no memory response was found against N. meningitidis after 8 years of vaccination in young adults (N. Rautonen et al., 1986, J. of Immunol. 137: 2670-2675).

The polysaccharide corresponding to N. meningitidis serogroup B is poorly immunogenic (E. Gotschlich et al., 1969, J. Exp. Med. 129: 1349-1365) and induces a poor response of IgM of low specificity (W. Zollinger et al., 1979, J. Clin. Invest. 63: 836-848). There are different theories related to this problem, such as cross-reactivity between B polysaccharide and fetal brain structures, antigenic structures modified in solution and sensitivity to neuroaminidases (C. Moreno et al., 1985, Infect. Immun. 47: 527-533). Recently, a chemical modification of PS B was achieved, which induced a response in the host (H. Jennings et al., 1988, US Patent 4 727 136; F. Ashton et al., 1989, Microb. Pathogen. 6: 455-458), but safety of this vaccine in humans has not been demonstrated.

Due to the lack of an effective vaccine against N. meningitidis B, and because the risk of endemic infection is low and mainly restricted to children, a routine immunization with polysaccharides is not recommended (C. Frasch, 1989, Clin. Microbiol. Revs. 2: S134-S138) except in the case of an epidemic.

Since after the Second World War the disease was caused in most of the cases by N. meningitidis B, vaccines against serogroup B gained special significance.

Other outer membrane components of N. meningitidis include phospholipids, lipopolysaccharides (LPS or endotoxins), pili proteins and others. Different immunotypes of LPS have been described for N. meningitidis (W. Zollinger and R. Mandrell, 1977, Infect. Immun. 18: 424-433; C.M. Tsai et al., 1983, J. Bacteriol. 155: 498-504) and immunogenicity using non-toxic derivatives was assayed (H. Jennings et al., 1984, Infect. Immun. 43: 407-412) but their variability (H. Schneider et al., 1984, Infect. Immun. 45: 544-549) and pyrogenicity (when it is conjugated to lipid A) are limiting factors up to now.

The pili, structures needed to fix cells to nasopharingeal mucous membrane (D. Stephens et al., 1983, The J. Infect. Dis. 148: 369-376) have antigenic diversity among different strains (J. Greenblatt et al., 1988, Infect. Immun. 56: 2356-2362) with some common epitopes (D. Stephens et al., 1988, The J. Infect. Dis. 158: 332-342). Presently there are some doubts in relation to the effectiveness of a vaccine based on these structures. However some of these types of vaccine have been obtained, without known results related to

their use in humans (C. Brinton, 1988, US Patent 4 769 240).

Recently, the attention has switched to the other proteins of the outer membrane of this bacterium. There are many immunological types of these protein complexes.

The strains of N. meningitidis are subdivided in serotypes according to the presence of specific epitopes in the majoritary protein P1/P2 and in subtypes according to other epitopes in protein P1 (C. Frasch et al., 1985, Rev. Infect. Dis. 7: 504-510).

There are several published articles and patent applications concerning vaccines based on cocktails of these proteins, with previous selective removal of endotoxins using biocompatible detergents. The immunogenicity of these cocktails in animals and humans has been demonstrated (W. Zollinger et al., 1979, J. Clin. Invest. 63: 836-848; C. Frasch and M. Peppler, 1982, Infect. Immun. 37: 271-280; E. Beuvery et al., 1983, Infect. Immun. 40: 369-380; E. Rosenqvist et al., 1983, NIPH Annals 6: 139-149; L. Wang and C. Frasch, 1984, Infect. Immun. 46: 408-414; C. Moreno et al., 1985, Infect. Immun. 47: 527-533; E. Wedege and L.

Froholm, 1986, Infect. Immun. 51: 571-578; C. Frasch et al., 1988, The J. Infect. Dis. 158: 710-718; M. Lifely. and Z. Wang, 1988, Infect. Immun. 56: 3221-3227; J. Poolman et al., 1988, In J. Poolman et al (Eds), Gonococci and Meningococci, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 159-165; E. Rosenqvist et al., 1988, J. Clin. Microbiol. 26: 1543-1548), including results in massive field trials e.g. Capetown, South Africa in 1981 (C. Frasch, 1985, Eur. J. Clin. Microbiol. 4: 533-536); Iquique, Chile, 1987 (W. Zollinger, 1988, Proceedings of the Sixth International Pathogenic Neisseria Conference. Callaway Gardens Conference Center) and Cuba 1986 and 1988 (G. Sierra, 1988, Proceedings of the Sixth International Pathogenic Neisseria Conference. Callaway Gardens Conference Center). However, with the exception of the last case, the bactericidal antibodies induced by these preparations were restricted to the same serotype strains or related ones.

One of these vaccines is referred to in US patent 4,601,903 which is restricted to one of the Neisseria types producing meningitis (serotype 2), with a high incidence, but also other serotypes have been isolated with high frequency from patients, such as serotypes 4 (Cuba from 1981 to 1983, H. Abdillahi et al., 1988, Eur. J. Clin. Microbiol. Infect. Dis. 7: 293-296; Finland from 1976 to 1987, H. Kayhty et al., 1989, Scand. J. Infect. Dis. 21: 527-535); 8 (Australia from 1971 to 1980, F. Ashton et al., 1984, Can. J. Med. Biol. 30: 1289-1291) and 15 (Norway from 1982 to 1984, L. Froholm et al., 1985, Proceedings of the Fourth International Symposium on Pathogenic Neisseria. American Society for Microbiology; Chile from 1985 to 1987, S. Ruiz et al., 1988, Proceedings of the Sixth International Pathogenic Neisseria Conference. Callaway Gardens Conference Center) as well as strains of undefined serotype (F. Ashton et al., 1980, Can. J. Microbiol. 26: 1480-1488; Australia from 1971 to 1980, F. Ashton et al., 1984, Can. J. Med. Biol. 30: 1289-1291; Finland from 1976 to 1987, H. Kayhty et al., 1989, Scan. J. Infect. Dis. 21: 527-535).

The Cuban vaccine achieved in 1988 by the Centro Nacional de Biopreparados (European Patent Application. No. 301 992) has proven to be very effective. It is based on a high molecular weight antigenic complex. It possesses a broad range of cross-reactivity with other strains and produces and maintains bactericidal antibodies in the immunized host.

However, the methods employed to obtain this type of vaccine start with the multiplication in an appropiate culture of a microorganism which is highly pathogenic, with the associated biological risk of handling directly the bacteria. Moreover, this kind of preparation contains lipopolysaccharides, a contaminant that, although it may increase the product's effectiveness, shows at the same time undesirable secondary effects because of its powerful pyrogenicity. Also, its variation in minor antigenic components, which form part of the preparation, cannot be controlled in the different batches, which makes it difficult to follow important parameters related to the reactogenicity and immunogenicity.

For this reason, there is increasing interest in the identification of nucleotide sequences coding for highly conserved proteins in all strains, and even more so the identification of inducer proteins of bactericidal antibodies common to the majority of pathogenic Neisseria, in order to obtain vaccine preparations with a broad spectrum of protection.

There are different proteins with high molecular weight which are present in low amounts in the outer membrane of N. meningitidis when this microorganism is grown in conventional culture media but have a strong response in affected individuals (J. Black et al., 1986, Infect. Immun. 54: 710-713; L. Aoun et al., 1988, Ann. Inst. Pasteur/ Microbiol. 139: 203-212) and/or increase their response under special culture conditions (J. van Putten et al., 1987, Antoine van Leeuwenhoek 53: 557-5564; A. Schryvers and L. Morris, 1988, Molecular Microbiol. 2: 281-288 and Infect. Immun. 56: 1144-1149). Some of these proteins are highly conserved among the different strains, in particular those related to the acquisition of iron by the microorganism, that have become interesting vaccine candidates (L. Mocca et al., 1988, Proceedings of the Sixth International Pathogenic Neisseria Conference. Callaway Gardens Conference Center; C. Frasch,

1989, Clin. Microbiol. Revs. 2: S134-S138).

In addition to pure proteins obtained from the micro-organism or strains of related species (e.g. 37 kD protein, T. Mietzner and S. Morse, 1987, US Patent 4 681 761), several related genes have been cloned and expressed. Among these proteins are the following:

protease IgAl (J. Koomey and S. Falkow, 1984, Infect. Immun. 43: 101-107);
protein P1 (A. Barlow et al., 1987, Infect. Immun. 55: 2734-2740, and 1989, Molec. Microbiol. 3: 131-139);
protein P5a (T. Kawula et al., 1988, Infect. Immun. 56: 380-386);
protein P5c (T. Olyhoek and M. Achtman, 1988, Proceedings of the Sixth International Pathogenic N. Conference. Callaway Gardens Conference Center);
protein P4 (K. Klugman et al., 1989, Infect. Immun. 57: 2066-2071);
protein P2 (K. Murakami et al., 1989, Infect. Immun. 57: 2318-2323);
and from N. gonorrhoeae, which code for proteins with cross-reactivity with their corresponding proteins from N. meningitidis:
antigen H.8 (W. Black and J.G. Cannon, 1985, Infect. Immun. 47: 322-325);
macromolecular complex (W. Tsai and C. Wilde, 1988, Proceedings of the Sixth International Pathogenic Neisseria Conference. Callaway Gardens Conference Center);
37 kDa protein, repressed in the presence of iron (S. Berish et al., 1988, Proceedings of the Sixth International Pathogenic Neisseria Conference. Callaway Gardens Conference Center).

The use of these proteins as active vaccine preparation has not been reported or the bactericidal tests of antibodies induced against them were negative, such as in the case of mouse monoclonal antibodies against H.8 (J. Woods et al., 1987, Infect. Immun. 55: 1927-1928).

Up to the moment, the protein P1 located in the outer membrane of N. meningitidis is one of the best characterized and studied antigens. This protein presents no variability within the same strain. However, there are more than 17 types of proteins P1 in Neisseria which have differences in three variable regions, this being the basis of the classification of N. in different subtypes. This protein is very immunogenic in humans (W.D. Zollinger and R.E. Mandrell, 1983, Med. Trop. 43:143-147), eliciting protective antibodies (E. Wedege and L.O. Froholm, 1986, Infect. Immun. 51: 571-578; K. Saukkonen et al, 1987, Microb. Pathogen. 3:261-267), that give it a special importance in vaccine preparations.

Some subtypes of proteins P1 have been cloned in E. coli, starting from genomic libraries (A.K. Barlow et al., 1989, Molec. Microb. 3:131-139) or using the PCR technique (S. Butcher et al., VIIth International Congress of Neisseria, R.C. Seid, Patent Application WO 90/06696; Brian Mc Guinness et al., 1990, J. Exp. Med. 171:1871-1882, M.C.J. Maiden et al., VIIth International Conference of Neisseria, Berlin, Sept. 9-14, 1990, and 1991, Molec. Microb. 3:727; J. Suker et al., VIIth International Conference of Neisseria, Berlin, Sept. 9-14, 1990). However, up to now, there is no genetic construction able to produce this protein with high levels of expression. Only low levels of expression (D.A. White et al., 1990, Molec. Microb. 4:769:776) or its expression in Bacillus subtilis fused to the outer membrane protein A of E. coli (omp A) (E. Wahlstrom et al., VIIth International Congress of Neisseria, September 9-14, 1990, Berlin) have been reported.

It can be affirmed that up to the moment no antigen has been isolated which is common to all types and serogroups of N. meningitidis and is able to produce bactericidal antibodies. For this reason, an antigen of this kind, conjugated or fused to other proteins or polysaccharides of immunological interest, would be relevant as a candidate for bivalent vaccine preparations.

This invention is related to a nucleotide sequence coding for a protein having a molecular weight of about 64 kilodaltons. This sequence has been found in all N. meningitidis serotypes and serogroups tested, as verified by nucleic acid hybridization, Western-bloting, Dot-blot and ELISA.

A technical object of this invention is the identification of a nucleotide sequence which codes for a highly conserved protein and is common to the majority of pathogenic strains of Neisseria (named P64k), in order to obtain the protein by a recombinant way with a high grade of purity and in commercially useful quantities, so that it can be employed in diagnostic methods and as an integrating part of a vaccine preparation of broad spectrum of protection.

On the level of genetic information (DNA and RNA), the invention provides a recombinant polynucleotide, comprising a nucleotide sequence coding for a protein P64k of Neisseria meningitidis, said protein P64k essentially having the amino acid sequence shown in SEQ ID NO:1. In a preferred embodiment said nucleotide sequence coding for the protein P64k of N. meningitidis essentially consists of the nucleotide sequence shown in SEQ ID NO:1. The recombinant polynucleotide may further comprise a nucleotide sequence of a cloning or expression vector.

The invention also provides a transformed microorganism containing a recombinant polynucleotide as defined above, preferably a transformed microorganism which is capable of expressing the protein P64k of N. meningitidis. In a particularly preferred embodiment of the invention, the transformed microorganism is

an Escherichia coli strain, e.g. E. coli strain HB101, transformed with an expression vector containing a nucleotide sequence coding for the protein P64k of N. meningitidis, e.g. the expression vector pM-6.

The invention also provides a recombinant proteinaceous substance, comprising an amino acid sequence corresponding to the amino acid sequence of at least a part of a protein P64k of N. meningitidis, said protein P64k essentially having the amino acid sequence shown in SEQ ID NO:1. Said recombinant proteinaceous substance may essentially consist of protein P64k, or be a fusion protein or a protein/polysaccharide conjugate comprising the amino acid sequence of protein P64k of N. meningitidis.

The invention further provides a vaccine composition, comprising a recombinant protein as defined above, together with a suitable carrier, diluent or adjuvant. A particular embodiment of this invention provides a vaccine composition, comprising a lipoamide dehydrogenase or acetyl transferase capable of inducing antibodies which can bind a protein P64k of N. meningitidis, together with a suitable carrier, diluent or adjuvant.

In addition, the invention provides a monoclonal antibody, raised against a recombinant proteinaceous substance as defined above, or against a lipoamide dehydrogenase or acetyl transferase, and capable of binding a protein P64k of N. meningitidis.

The invention also provides a process for preparing a protein P64k of Neisseria meningitidis, or a fusion protein comprising protein P64k, said protein P64k essentially having the amino acid sequence shown in SEQ ID NO:1, comprising the steps of transforming a microorganism with an expression vector containing a nucleotide sequence coding for said protein P64k, or said fusion protein, culturing the transformed microorganism to obtain expression of said protein P64k, or said fusion protein, and isolating said expression product.

One novel aspect of this invention is the gene isolated from the N. meningitidis strain B:4:P1.15, which was named M-6 and has as a principal characteristic its stability in E. coli vectors. This gene does not produce adverse effects on the host, allowing to obtain yields of over 25 % of total protein (ratio of P64k to total protein from host strain). On the other hand, it has been demonstrated by Southern and Western Blot hybridizations (E. Southern, 1975, J. Mol. Biol. 98: 503-527 and W. Burnette, 1981, Anal. Biochem. 12: 195-203) that protein P64k is present in all the following studied strains of N. meningitidis:

N. meningitidis A
N. meningitidis B:1
N. meningitidis B:2
N. meningitidis B:4
N. meningitidis B:5
N. meningitidis B:8
N. meningitidis B:9
N. meningitidis B:11
N. meningitidis B:15
N. meningitidis B:4:P1.15
N. meningitidis C
N. meningitidis B:15:P1.16
N. meningitidis B:15:P1.16 (H 44/76)
N. meningitidis B:NT (121/85)
N. meningitidis B:NT (71/86)
N. meningitidis B:NT (210/86)

and also in N. mucosa, N. subflava and N. gonorrhoeae.

The protein is not present in N. cinerea, N. lactamica, N. sicca and N. flavescens, but these are not of interest because they are not pathogenic.

The protein having a molecular weight of about 64 kDa can be localized by electron microscopy in the outer membrane of N. meningitidis. Therefore, this antigen is an exposed antigen which is favorable for use in a vaccine preparation. The protein was recognized in Western blot immunoidentification experiments with sera from convalescents and individuals vaccinated with the conventional Cuban vaccine Va-Mengoc-BC (Centro Nacional de Biopreparados, Havana, Cuba). This aspect guarantees the immunogenicity of the antigen and at the same time confirms its presence within the high molecular weight protein fraction constituent of this vaccine, which is responsable of the lasting immune response to the disease.

Another novel aspect is that the protein, which is an object of this invention, produces antibodies with a broad bactericidal spectrum (different serogroupes, serotypes and subtypes), a characteristic which has not been reported previously for any protein from N. meningitidis.

This protein obtained in high levels in E. coli becomes an important candidate for the improvement of immunogenicity when expressed as a fusion protein with other proteins. It could also increase the

expression by conferring enhanced stability and suitability in the molecular structure during transcription and translation processes. Belonging to Neisseria, this protein can also be fused to other proteins from Neisseria in order to obtain vaccine preparations against this microorganism with increased immunogenicity. These fusion proteins are also objects of this invention.

On the other hand, surprisingly, it was found that the gene M-6 obtained from a genomic library of the strain N. meningitidis B:4:P1.15 showed a great homology with sequences of lipoamide-dehydrogenases and acetyl-transferases from other microorganisms and higher organisms. The presence of common antigenic determinants allows the use of these other related proteins as immunogens, able to confer protection by the induction of bactericidal antibodies which recognize the antigenic determinants common to protein P64k. Therefore, the use of these lipoamide-dehydrogenases and acetyl-transferases (not isolated from N. meningitidis) or derivatives therefrom such as peptides, fragments from enzymatic degradation, constructions of fusion with other proteins, or conjugation with proteins, polysaccharides or lipids, or insertion in complexes as liposomes or vesicles, etc., for vaccine purposes, are included in the scope of this invention.

An important object of this invention is the nucleotide sequence which codes for the M-6 gene (SEQ ID NO:1 of the Sequence Listing) whose product is the protein P64k.

This gene was derived from the genome of the strain B385 isolated in Cuba (N. meningitidis B:4:P1.15), by the construction of a genomic library in the phage EMBL 3.

The recombinant DNA including the gene M-6 constitutes another object of this invention, which includes the phage lambda, the plasmid pM-3 and the expression vector pM-6 for expression in bacteria.

In particular, for the intracellular expression in E. coli, the M-6 gene was cloned under the tryptophane promotor and using its own termination signal of transcription and a linkage fragment between M-6 and the cloning site NcoI which adds the following nucleotide sequence at the 5' end:

ATG CTA GAT AAA AGA (SEQ ID NO:2)

The N-terminal of the protein P64k encoded by the M-6 gene inserted in plasmid pM-6 which adds 5 aminoacids to the N-terminal of the original protein corresponds to:

M L D K R M A L V E L K V P D I G G H E N V D I I (SEQ ID NO:3)

Another object of this invention are the microorganisms resulting from the transformation of E. coli strain HB 101 with the pM-6 vector, which are characterized by the expression of high levels of protein P64k, good viability and great stability.

The transformed clone of E. coli was denominated HBM64 (Fig. 2), and presents levels of expression of P64k higher than 25 % in relation to the total protein of the cell (Fig. 6).

The procedure described in the present invention, due to the levels of expression achieved for this product, allows to reach an optimal purity for use of this protein in humans.

On the other hand, the antigen obtained from the isolated sequence was very useful in the preparation of different types of potential vaccine preparations, like bivalent vaccines with a broad immunoprotective spectrum, e.g., protein-polysaccharide conjugates, fusion proteins, etc.

EXAMPLES: These examples intend to illustrate the invention, but not to limit the scope of this invention.

EXAMPLE 1:

For the isolation of genomic DNA from N. meningitidis B:4:P1.15, the cells were grown in Mueller-Hinton medium (OXOID, London). The biomass from a culture of 100 ml was resuspended in 8 ml of Tris (hydroxymethyl-aminomethane) 100 mM, EDTA (ethylenediamine tetraacetic acid) 1mM, pH 8. The cells were subjected to a treatment with lysozyme (10 mg/ml), followed by 200 µl of self-digested pronase (20 mg/ml) and 1.1 ml of 10% SDS. The mixture was incubated at 37° C during 1 hour, then it was treated with phenolchloroform (v/v) and the remains of phenol were eliminated using 2-butanol. Finally, the DNA was precipitated with absolute ethanol and RNA was eliminated with ribonuclease A (Sigma, London).

The DNA of about 60 kb was subjected to a partial digestion with the enzyme Sau 3A, obtaining a population of fragments of about 15 kb. This majoritary fraction was isolated and purified by separation in agarose gel (T. Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.: Cold Spring Harbor NY).

For the construction of the genomic library, the process described by Maniatis was essentially followed (T. Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.: Cold Spring Harbor NY). Four µg of purified DNA were ligated with 8 µg of BamHI-digested EMBL-3. The ligation product was packed and the phages were finally plated on the E. coli strain C66P2.

The library was screened by immunoidentification (R. Young and R. Davis, 1983, PNAS USA 80: 1194-

1198) using rabbit serum obtained against a preparation of proteins belonging to the outer membrane of the strain N. meningitidis B:4:P1.15. The clones were analyzed by Western-blot (Burnette, 1981) and the expression of the P64k protein with a molecular weight of about 70 kDa was detected. The resulting recombinant phage was named 31. The Western blot was also made using a mixture of sera from convalescents of meningococcemia, free of antibodies from E. coli, obtaining the same result as that using hyperimmunized rabbit sera.

This experiment was repeated using sera from several healthy individuals, and the signal obtained was negative against the recombinant protein P64k.

EXAMPLE 2:

For subcloning in bacteria, the 17 kb insert corresponding to the phage isolated from the library was cloned in the plasmid pUC18 after separation from the phage's arms using the enzyme SalI. This resulted in the construction pM-1 (Fig. 2), that was subjected to restriction analysis (Fig. 3).

The fragment SalI-HindIII of about 6 kb was recloned in the plasmid pUC18 and the construction pM-2 was obtained (Fig. 2). In order to obtain a more exact localization of the gene coding for protein P64k deletions were carried out with the enzymes ClaI, EcoRI and HincII. The complete fragment of the gene M-6 was finally localized as an EcoRI-HindIII insert corresponding to the construction pM-3 (Fig. 2).

In all constructions, the presence of the gene was confirmed by recognizing the protein by colony immunoidentification and Western Blot using hyperimmunized rabbit sera.

The sequence of the insert in pM-3 was determined by the method of Sanger (F. Sanger et al., 1977, PNAS USA 74: 5463-5467).

From the obtained sequence, the approximate molecular weight of the protein encoded by the gene was deduced.

In order to obtain a construction for high expression of the protein P64k, the plasmid pM-3 (Fig. 2) was linearized with the enzyme EcoRI and successive suppressions of the gene were carried out, incubating the sample with the nucleases ExoIII and S1.

The resulting fragments were separated from the rest of the vector pUC18 by cutting with the restriction enzyme HindIII and were cloned fused to a stabilizator fragment (European patent application EP-A-0 416 673), using an Xba-blunt adapter to conserve the XbaI site of the stabilizator gene:

```
5' C T A G A T A A A A G A  3'          (SEQ ID NO:4)

3'         T A T T T T C T  5'          (SEQ ID NO:5)
```

The constructions in which the fused fragment coincided with the reading frame were selected by immunoidentification using hyperimmune rabbit sera.

The insert sequences were established using Sanger's Method (F. Sanger et al., 1977, PNAS USA 74: 5463-5467). From the obtained sequences the approximate molecular weight of the protein encoded by this gene was deduced.

The fusion region between the proteins was localized in the gene sequences. In the clone pILM-25 (Figure 4) the ATG of the gene predetermined by the sequence of the DNA insert isolated from the library, coincided with the fusion site.

The NcoI-XbaI fragment, corresponding to the stabilizing peptide coding sequence, was deleted from pILM-25, obtaining a non-fused protein expressed under the tryptophan promoter with it's original terminator from the N. Meningitidis B:4:P1.15, according to the pM-6 construction (Figure 1).

The pM-6 plasmid was transformed in different strains of E. coli like W3110, JA-221, HB-101, LE-392 and MC-1061, and the expression of P64k was compared. The best results were obtained in W3110, JA-221 and HB-101. These strains were chosen to scale up fermentation, and expression levels up to 25 % of total cell proteins were obtained.

EXAMPLE 3:

To confirm the correct expression of the cloned gene the N-terminal region of the intact protein was subjected to the Edman degradation method (P. Edman, 1950, Acta Chem. Scand. 4: 283-293). This

technique elucidates the sequence (primary structure) of this region in the molecule.

The P64k protein was desalted by gel filtration chromatography (PD-10, Pharmacia), eluted with water and monitored at 280 nm. The protein fraction was concentrated to 0.5 nM/µl. One µl of this solution was applied to a PVDF (polyvinylidene difluoride, Millipore) filter, previously activated with methanol.

The Edman degradation was made using the Knauer's Automatic Sequencer, model 810, connected to a HPLC (High Performance Liquid Chromatography) system, so as to detect the phenylthiohydantoin derivatives of the aminoacids (PTH-aminoacids). The standard procedure of sequencing as recommended by the manufacturer of the equipment was followed. The separation of the PTH-aminoacids was performed in a reverse phase column C-18 (5 µm), 250 mm x 2 mm (Merck), eluted with an acetonitrile gradient (B buffer) in sodium acetate (A buffer), prepared according to the manufacturers, with a 200 µl/minute flow and at 42 ° C. The PTH-aminoacids were detected at 269 nm.

Data processing and registration were made in a Shimadzu model CR-6a automatic integrator, using a program for data processing by subtraction of two consecutive chromatograms, to facilitate the evaluation of the Edman degradation cycles. Sequence identification is obtained by the chromatographic evaluation of the corresponding analyzed cycle and confirmed by the chromatogram obtained by subtraction, allowing to determine 25 residues.

EXAMPLE 4:

To demonstrate that the protein P64k is recognized by the sera of individuals vaccinated with the Cuban Va-Mengoc-BC preparation (Centro Nacional de Biopreparados, Havana, Cuba.), a Western-Blot was made, with a mixture of 12 sera from adults (immunized with two doses of the Cuban vaccine) diluted in a solution containing defatted milk (Oxoid, London). The experiment included:
recombinant protein P64k, purified from E. coli HB-101 transformed with the pM-6 plasmid;
supernatant of the ultrasonic cell rupture of untransformed E. coli HB-101;
the reaction was revealed with a protein A-colloidal gold conjugate.
It was shown that the protein P64k is recognized by the pool of sera.

EXAMPLE 5:

The bactericidal test against B385 (B:4:P1.15) was made according to the procedure described by Larrick et al. (Scand. J. Immunol. 32, 1990, 121-128) with modifications. With this objective, a mixture was made of a) a suspension of bacteria, cultured under special conditions (1-5 colony forming units/1), b) Gey's balanced salt solution, c) rabbit sera (3 to 4 weeks) as a source of complement and d) pooled sera from mice, immunized against protein P64k in Aluminium Hydroxyde Gel, and inactivated at 56 ° C for 30 minutes. The immunization of mice was carried out according to an immunization scheme of 3 doses of 20 µg each. The proportions used in the aforementioned mixture were 1:2:1:1 in a total volume of 125 µl. The mixture was incubated at 37 ° C during 1 hour and plated in fresh Mueller Hinton Agar (Oxoid, London) supplemented with 5 % calf serum (CubaVet, Habana). The counting of surviving colonies was done after 18 hours of incubation of the plates in an atmosphere of 5 % $CO_2$ at 37 ° C.

The bactericidal titer was considered as the maximum serum dilution necessary to render a 50 % inhibition of bacterial growth, with respect to the same mixture without the test serum. It was found that 1:20 serum dilution still maintains its bactericidal activity. As negative controls (non bactericidal at 1:2 dilution) pooled sera from mice immunized with Aluminum Hydroxyde Gel, and pooled sera from mice immunized with cuban Hepatitis B recombinant vaccine, were used. The bactericidal effect was specific to the anti-P64k antibodies.

EXAMPLE 6:

The bactericidal test against different strains of N. meningitidis was made using:

1. An ammonium sulphate precipitate of the supernatant harvested from a culture of hybridoma cells secreting monoclonal antibodies specific against P64k (anti P64k)/Sample to analyze.
2. An ammonium sulphate precipitate of the supernatant harvested from hybridoma cells secreting monoclonal antibodies specific against the P1.15 protein present in N. meningitidis strain B385 (anti P1.15)/Positive control of the system.

The maximum dilutions tested were always 1:16. The maximum dilutions tested which had a bactericidal effect, according to the EXAMPLE 5, are indicated:

| Strain | anti-P64k | anti-P1.15 |
|---|---|---|
| B385 | 1:16 | 1:16 |
| B:4:P1.15 | 1:16 | 1:16 |
| B:14:P1.7 | 1:16 | - |
| B:NT:NT | 1:16 | - |
| B:15:P1.15 | 1:8 | - |
| B:15:P1.16 | 1:8 | - |
| B:13 | 1:8 | - |
| C | 1:16 | - |
| A | 1:16 | - |

As seen, the anti-P64k monoclonal antibodies have significant bactericidal titers against different serogroups (A, B and C), serotypes (4, 14, 13, 15 and NT) and subtypes (7, 15, 16 and NT) of bacteria.

EXAMPLE 7:

Fusion protein M-14 (P64k and P1.15)

In order to obtain a genetic construction for high expression that contained the variable epitopes of the P1.15 protein (Outer membrane protein from N. meninigitidis B:4:P1.15) fused to the P64k protein, the gene coding for P1.15 protein was cloned using the Polymerase Chain Reaction (PCR). The following region containing the variable immunodeterminants of P1.15:

L Q L T E P P S K S Q P Q V K V T K A K S R I R T K I S D F G
S F I G F K G S E D L G E G L K A V W Q L E Q D V S V A G G G
A T Q W G N R E S F V G L A G E F G T L R A G R V A N Q F D D
A S Q A I D P W D S N N D V A S Q L G I F K R H D D M P V S V
R Y D S P D F S G F S G S V Q F V P I Q N S K S A Y T P A Y H
Y T R Q N N A D V F V P A V V G K P G S D V Y V A G L N Y K N
G G F A G S Y A F K Y A R H A N V G R N A F E L F L L G S T S
D E A (SEQ ID NO:6)

was inserted in the Mlu I site of the gene M-6, encoding for P64k, after having been made blunt with the klenow fragment from DNA polymerase I. The sites for gene fusion of P1.15 with M-6 are the following:

GDALQL (SEQ ID NO:7)

Gly Asp Ala Leu Gln Leu
5'- GGC GAC GCG CTG CAG TTGA -3' (SEQ ID NO:8)
M-6 P1.15

EANAYE (SEQ ID NO:9)

Glu Ala Asn*Ala Tyr Glu

5'- GAA GCC AAC GCG TAC GAA -3' (SEQ ID NO:10)

P1.15 M-6

*: N does not belong to any of the fusion proteins and was created by the genetic construction.

The resulting fusion protein (M-14) was expressed in E. coli using a plasmid vector under the tryptophan promoter, to levels higher than 10 % of total cell protein. The protein was recognized by bactericidal monoclonal antibodies, and anti-P1.15 and P64k polyclonal antibodies, in Western-Blot.

EXAMPLE 8:

Polysaccharide/P64k conjugation.

The protein P64k was conjugated with the polysaccharide from Haemophilus influenzae using the reductive amination method. The Haemophilus influenzae polysaccharide (Polyribosyl ribitol phosphate, PRP) was purified by the cold phenol method described by Frasch, 1990 (in: Bacterial Vaccines, 1990, Alan R. Liss, Inc., pp. 123-145). The final contamination of PRP with proteins or nucleic acids was less than 1 %. This polysaccharide was degraded using the method of Parikh et al. 1974 (Methods in Enzymol. 34B: 77-102) with sodium periodate in PRP (ratio 1 : 5 w/w) dissolved in 0.1 M sodium acetate (pH 4.5). The incubation was carried out in the dark during 30 minutes with stirring. The periodate excess was eliminated by addition of ribitol. Very low molecular weight compounds were eliminated by dialysis (Medicell International Ltd. Membrane, London). The resulting oligosaccharide had free aldehyde groups able to react with primary amines (e.g. lysine residues in proteins). The conjugate is obtained by mixing protein and polyssacharide in a 1 : 1 ratio (w/w), adding sodium cyanoborohydride and subjecting the mixture to an incubation, first for 48 hours at 4 °C and later at 37 °C for 24 hours. The high molecular weight complex which contains the resulting conjugate with protein-polysaccharide in a 1 : 2.3 ratio, can be separated from the non reactive contaminants by HPLC.

EXAMPLE 9:

Bivalent vaccine preparation against Hepatitis B virus and N. meningitidis.

In order to obtain a bivalent vaccine preparation, different quantities of protein P64k and Hepatitis B Surface Antigen (Vacuna Recombinante contra 1a Hepatitis B, Heber Biotec, Havana, Cuba) were mixed. The antigens were adjuvated with Aluminum Hydroxyde Gel, at 2mg/dose and inoculated in Balb/c mice having a body weight of 20 g in 3 dosis of 0.5 ml each. Different variants were assayed:

1. P64k 20 µg (P20)
2. HBsAg 20 µg (H20)
3. P64k 10 µg + HBsAg 10 µg (P10:H10)
4. P64K 15 µg + HBsAg 5 µg (P15:H5)
5. Placebo ($Al(OH)_3$)

Seven days after the immunization with the first doses, the second doses were applied. The third dose was given 14 days after the second. Seven days later, blood was taken and the serum of each immunized animal was separated. Antibody titers against P64k protein were measured in solid phase Enzyme Linked Immunosorbent Assay (ELISA), using P64k at 5 mg/ml to coat the polystyrene plate. The antibody titers against HBsAg were determined by a Commercial ELISA (Organon Teknika, Boxtel). Figure 5 shows the dynamics of antibody response against protein P64k, using sera diluted 1/10 000. The response against P64k is not interfered by the presence of the other antigen. Figure 6 shows the titers against HBsAg after each dosis. The titers against this protein are not diminished by the presence of P64k in the preparation.

High titers are obtained against both antigens in the same vaccine preparation.

EXAMPLE 10:

A software was created to search the EMBL (European Molecular Biology Laboratoty) Data Base and detect the homology between P64k and other proteins. As result of the search it was found that there is homology of one segment in the sequence of P64k with segments in the sequences of N. gonorrhoeae. This sequence was found as characteristic in both N. gonorrhoeae and N. meningitidis (F.F. Correia, S. Inouye and M. Inouye, 1988, J. Biol. Chem. 263, No. 25, 12194-12198).

Another region with high homology was found in two proteins of the Pyruvate Dehydrogenase Complex from E.coli K12:

a)Acetyltransferase from E. coli and the P64k outer membrane protein from N. meningitidis.

Homology exists between a segment comprising 100 amino acids, repeated at the beginning of the amino acid sequence of the Acetyltransferase ("Lipoyl Domain", including the "Lipoyl Binding Site" (P.E. Stephens et al., 1983,. Eur. J. Biochem. 133, 481-489)) and a region located in the first 111 amino acids of the P64k:

```
MALVELKVPDIGGHENVDIIAVEVNVGDTIAV          (SEQ ID NO:11)
  - *- ****** - *--  * * *** -*
  VKEVNVPDIGG DEVEVTEVMVKVGDKVAA          (SEQ ID NO:12)

DDTLITLETDKATMDVPAEVAGVVKEVKVKVG          (SEQ ID NO:11)(cont)
-- ***-* *** *-***  ******-** **
EQSLITVEGDKASMEVPAPFAGVVKELKVNVG          (SEQ ID NO:12)(cont)

DKISEGGLIVVVEAEGT--AAAPKAESAA--A          (SEQ ID NO:11)(cont)
**-  * **-- * **   **** - **  *
DKVKTGSLIMIFEVEGAAPAAAPAKQEAAAPA          (SEQ ID NO:12)(cont)

PRKKPLKCRWVPQAAQFGG                       (SEQ ID NO:11)(cont)
*  *       * *  *
PAAKAEAPAAAPAAKAEGK                       (SEQ ID NO:12)(cont)
```

where (*) indicates positions with the same amino acids and (-) indicates positions of conservative amino acid changes.

b) Lipoamide Dehydrogenase from E. coli and Outer Membrane P64k protein from N. meningitidis.

Homology exists between the Lipoamide Dehydrogenase from E. coli (a protein having 473 amino acids, P.E. Stephens et al., 1983, Eur. J. Biochem. 133, 481-489) and the protein P64k, specifically in a segment which represents almost the total protein, except the region with homology with the "lipoyl domain" from Acetyltransferase.

```
                |---1----|
SADAEYDVVVLGGGPGGYSAAFAAADEGLKVA          (SEQ ID NO:13)
*-- - -******-**-******  ** **---
STEIKTQVVVLGAGPAGYSAAFRCADLGLETV          (SEQ ID NO:14)

    |-------2-------|
IVERYKTLGGVCLNVGCIPSKALLHNAAVIDE          (SEQ ID NO:13)(cont)
*****-******************** * **-*
IVERYNTLGGVCLNVGCIPSKALLHVAKVIEE          (SEQ ID NO:14)(cont)

VRHLAANGIKYPEPALDIDMLRAYKDGVVSRL          (SEQ ID NO:13)(cont)
-- **--** - **  ***--*--*- *---*
AKALAEHGIVFGEPKTDIDKI TWKEKVINQL          (SEQ ID NO:14)(cont)

TG-FGRYGEKRKVDVIQGDGQFLDPHHLEVSL          (SEQ ID NO:13)(cont)
** --  -- ***-*--* *-* --- ***-
TGGLAGMAKGRKVKVVNGLGKFTGANTLEVEG          (SEQ ID NO:14)(cont)

TAGDAYEQAAPTGEKKIVAFKNCIIAAGSRVT          (SEQ ID NO:13)(cont)
--*           *----*-* ******* -
ENG----------KTVINFDNAIIAAGSRPI           (SEQ ID NO:14)(cont)

KLPFIP-EDPRIIDSSGALALKEVPGKLLIIG          (SEQ ID NO:13)(cont)
-***** ***** **--**-******--**--*
QLPFIPHEDPRIWDSTDALELKEVPERLLVMG          (SEQ ID NO:14)(cont)

GGIIGLEMGTVYSTLGSRLDVVEMMDGLMQGA          (SEQ ID NO:13)(cont)
*********** -***--*****-* ----*
GGIIGLEMGTVYHALGSQIDVVEMFDQVIPAA          (SEQ ID NO:14)(cont)

DRDLVKVWQKQNEYRFDNIMVNTKTVAVEPKE          (SEQ ID NO:13)(cont)
*-*-***- *- - -*- -*--**--***-**
DKDIVKVFTKRISKKFN-LMLETKVTAVEAKE          (SEQ ID NO:14)(cont)

DGVYVTFEGANPPKEPQRYDAVLVAAGRAPNG          (SEQ ID NO:13)(cont)
**-***-** --* ********** **-***
DGIYVTMEGKKAPAEPQRYDAVLVAIGRVPNG          (SEQ ID NO:14)(cont)

KLISAEKAGVAVTDRGFIEVDKQMRTNVPHIY          (SEQ ID NO:13)(cont)
* --*-****-*-***** ****-*******-
KNLDAGKAGVEVDDRGFIRVDKQLRTNVPHIF          (SEQ ID NO:14)(cont)
```

```
AIGDIVGQPMLAHKAVHEGHVAAENCAGTKAY        (SEQ ID NO:13)(cont)
**************_*********  **_* *
AIGDIVGQPMLAHKGVHEGHVAAEVIAGKKHY        (SEQ ID NO:14)(cont)

FDAAVIPGVAYTSPEVAWVGETELSAKRPAGK        (SEQ ID NO:13)(cont)
**_ ***__***_******* ** _** _ _
FDPKVIPSIAYTEPEVAWVGLTEKEAKEKGIS        (SEQ ID NO:14)(cont)

ITKANFPWAASGRAIANGCDKPFTKLIFDAET        (SEQ ID NO:13)(cont)
 *_***********__*__ _****** *_
YETATFPWAASGRAIASDCADGMTKLIFDKES        (SEQ ID NO:14)(cont)

GRIIGGGIVGPNGGDMIAKSALPSKLGCDAAD        (SEQ ID NO:13)(cont)
 *_***_***_***_____ _*_ __****_*
HRVIGGAIVGTNGGELLGEIGLAIEMGCDAED        (SEQ ID NO:14)(cont)

     |--3-|
VGKTIHPRPTLGESIGMAAEVALGTCTDLPPQ        (SEQ ID NO:13)(cont)
__ ***__*** **_*_****  *_ **** _
IALTIHAHPTLHESVGLAAEVFEGSITDLPNP        (SEQ ID NO:14)(cont)

--KKK   -   MEN1pd                      (SEQ ID NO:13)(cont)
  ***
KAKKK   -   EC1pd                       (SEQ ID NO:14)(cont)
```

Where:
|-1-|: Adenine binding site (FAD)
|-2-|: Redox active disulphide region
|-3-|: Active site histidine

Strain deposits:

An E. coli HB-101 clone containing the plasmid pM-3 (a pUC18 plasmid containing the 4.1 kb DNA fragment from Neisseria meninigitidis, strain B:4:P1.15, cloned between the EcoRI and HindIII restriction sites), was deposited on August 30, 1991, with the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands, and obtained deposit number CBS 485 .91.

SEQUENCE LISTING

SEQ ID NO:1

SEQUENCE TYPE : Nucleotide with corresponding amino acid

SEQUENCE LENGTH: 1830 bp

STRANDEDNESS: Single

TOPOLOGY: Linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE ORGANISM: N. meningitidis group B

IMMEDIATE EXPERIMENTAL SOURCE: Strain B:4:P1:15 isolated in Cuba

FEATURES: From 1 to 1830 bp mature protein

PROPERTIES: Gene coding for P64k protein from outer membrane of N. meningitidis

```
ATG CTA GAT AAA AGA ATG GCT TTA GTT GAA TTG AAA GTG CCC    42
Met Leu Asp Lys Arg Met Ala Leu Val Glu Leu Lys Val Pro
  1               5                  10

GAC ATT GGC GGA CAC GAA AAT GTA GAT ATT ATC GCG GTT GAA    84
Asp Ile Gly Gly His Glu Asn Val Asp Ile Ile Ala Val Glu
 15                  20                  25

GTA AAC GTG GGC GAC ACT ATT GCT GTG GAC GAT ACC CTG ATT   126
Val Asn Val Gly Asp Thr Ile Ala Val Asp Asp Thr Leu Ile
     30                  35                  40

ACT TTG GAA ACC GAT AAA GCG ACT ATG GAC GTA CCT GCT GAA   168
Thr Leu Glu Thr Asp Lys Ala Thr Met Asp Val Pro Ala Glu
         45                  50                  55

GTT GCA GGC GTA GTC AAA GAA GTT AAA GTT AAA GTC GGC GAC   210
Val Ala Gly Val Val Lys Glu Val Lys Val Lys Val Gly Asp
             60                  65                  70

AAA ATC TCT GAA GGT GGT TTG ATT GTC GTC GTT GAA GCT GAA   252
Lys Ile Ser Glu Gly Gly Leu Ile Val Val Val Glu Ala Glu
                 75                  80

GGC ACG GCA GCC GCT CCT AAA GCC GAA TCG GCT GCC GCC CCG   294
Gly Thr Ala Ala Ala Pro Lys Ala Glu Ser Ala Ala Ala Pro
 85                  90                  95
```

```
CGC AAG AAG CCC CTA AAC GTG CCG CTC CCT GCT CCG CAA GCC   336
Arg Lys Lys Pro Leu Asn Val Pro Leu Pro Ala Pro Gln Ala
    100                 105                 110

GCG CAA TTC GGC GGT TCT GCC GAT GCC GAG TAC GAT GTG GTC   378
Ala Gln Phe Gly Gly Ser Ala Asp Ala Glu Tyr Asp Val Val
        115                 120                 125

GTA TTG GGT GGC GGT CCC GGC GGT TAC TCC GCT GCA TTT GCC   420
Val Leu Gly Gly Gly Pro Gly Gly Tyr Ser Ala Ala Phe Ala
            130                 135                 140

GCT GCC GAT GAA GGC TTG AAA GTC GCC ATC GTC GAA CGT TAC   462
Ala Ala Asp Glu Gly Leu Lys Val Ala Ile Val Glu Arg Tyr
                145                 150

AAA ACT TTG GGC GGC GTT TGC CTG AAC GTC GGC TGT ATC CCT   504
Lys Thr Leu Gly Gly Val Cys Leu Asn Val Gly Cys Ile Pro
155                 160                 165

TCC AAA GCC TTG TTG CAC AAT GCC GCC GTT ATC GAC GAA GTG   546
Ser Lys Ala Leu Leu His Asn Ala Ala Val Ile Asp Glu Val
    170                 175                 180

CGC CAC TTG GCT GCC AAC GGT ATC AAA TAC CCC GAG CCG GAA   588
Arg His Leu Ala Ala Asn Gly Ile Lys Tyr Pro Glu Pro Glu
        185                 190                 195

CTC GAC ATC GAT ATG CTT CGC GCC TAC AAA GAC GGC GTA GTT   630
Leu Asp Ile Asp Met Leu Arg Ala Tyr Lys Asp Gly Val Val
            200                 205                 210

TCC CGC CTC ACG GGC GGT TTG GCA GGT ATG GCG AAA AGC CGT   672
Ser Arg Leu Thr Gly Gly Leu Ala Gly Met Ala Lys Ser Arg
                215                 220

AAA GTG GAC GTT ATC CAA GGC GAC GGG CAA TTC TTA GAT CCG   714
Lys Val Asp Val Ile Gln Gly Asp Gly Gln Phe Leu Asp Pro
225                 230                 235

CAC CAC TTG GAA GTG TCG CTG ACT GCC GGC GAC GCG TAC GAA   756
His His Leu Glu Val Ser Leu Thr Ala Gly Asp Ala Tyr Glu
    240                 245                 250

CAG GCA GCC CCT ACC GGC GAG AAA AAA ATC GTT GCC TTC AAA   798
Gln Ala Ala Pro Thr Gly Glu Lys Lys Ile Val Ala Phe Lys
        255                 260                 265

AAC TGT ATC ATT GCA GCA GGC AGC CGC GTA ACC AAA CTG CCT   840
Asn Cys Ile Ile Ala Ala Gly Ser Arg Val Thr Lys Leu Pro
            270                 275                 280
```

```
TTC ATT CCT GAA GAT CCG CGC ATC ATC GAT TCC AGC GGC GCA   882
Phe Ile Pro Glu Asp Pro Arg Ile Ile Asp Ser Ser Gly Ala
                285                 290

TTG GCT CTG AAA GAA GTA CCG GGC AAA CTG CTG ATT ATC GGC   924
Leu Ala Leu Lys Glu Val Pro Gly Lys Leu Leu Ile Ile Gly
295                 300                 305

GGC GGC ATT ATC GGC CTC GAG ATG GGT ACG GTT TAC AGC ACG   966
Gly Gly Ile Ile Gly Leu Glu Met Gly Thr Val Tyr Ser Thr
    310                 315                 320

CTG GGT TCG CGT TTG GAT GTG GTT GAA ATG ATG GAC GGC CTG  1008
Leu Gly Ser Arg Leu Asp Val Val Glu Met Met Asp Gly Leu
        325                 330                 335

ATG CAA GGC GCA GAC CGC GAT TTG GTA AAA GTA TGG CAA AAA  1050
Met Gln Gly Ala Asp Arg Asp Leu Val Lys Val Trp Gln Lys
            340                 345                 350

CAA AAC GAA TAC CGT TTT GAC AAC ATT ATG GTC AAC ACC AAA  1092
Gln Asn Glu Tyr Arg Phe Asp Asn Ile Met Val Asn Thr Lys
                355                 360

ACC GTT GCA GTT GAG CCG AAA GAA GAC GGC GTT TAC GTT ACC  1134
Thr Val Ala Val Glu Pro Lys Glu Asp Gly Val Tyr Val Thr
365                 370                 375

TTT GAA GGC GCG AAC GCC CCT AAA GAG CCG CAA CGC TAC GAT  1176
Phe Glu Gly Ala Asn Ala Pro Lys Glu Pro Gln Arg Tyr Asp
    380                 385                 390

GCC GTA TTG GTT GCC GCC GGC CGC GCG CCC AAC GGC AAA CTC  1218
Ala Val Leu Val Ala Ala Gly Arg Ala Pro Asn Gly Lys Leu
        395                 400                 405

ATC AGC GCG GAA AAA GCA GGC GTT GCC GTA ACC GAT CGC GGC  1260
Ile Ser Ala Glu Lys Ala Gly Val Ala Val Thr Asp Arg Gly
            410                 415                 420

TTC ATC GAA GTG GAC AAA CAA ATG CGT ACC AAT GTG CCG CAC  1302
Phe Ile Glu Val Asp Lys Gln Met Arg Thr Asn Val Pro His
                425                 430

ATC TAC GCC ATC GGC GAC ATC GTC GGT CAG CCG ATG TTG GCG  1344
Ile Tyr Ala Ile Gly Asp Ile Val Gly Gln Pro Met Leu Ala
435                 440                 445

CAC AAA GCC GTT CAC GAA GGC CAC GTT GCC GCC GAA AAC TGC  1386
His Lys Ala Val His Glu Gly His Val Ala Ala Glu Asn Cys
    450                 455                 460
```

```
GCC GGC AAC AAA GCC TAC TTC GAC GCA CGG GTG ATT CCG GGC  1428
Ala Gly Asn Lys Ala Tyr Phe Asp Ala Arg Val Ile Pro Gly
        465                 470                 475

GTT GCC TAC ACT TCC CCC GAA GTG GCG TGG GTG GGC GAA ACC  1470
Val Ala Tyr Thr Ser Pro Glu Val Ala Trp Val Gly Glu Thr
            480                 485                 490

GAA CTG TCC GCC AAA GCC TCC GCG CGC AAA ATC ACC AAA GCC  1512
Glu Leu Ser Ala Lys Ala Ser Ala Arg Lys Ile Thr Lys Ala
                495                 500

AAC TTC CCG TGG GCG GCT TCC GGC CGT GCG ATT GCC AAC GGT  1554
Asn Phe Pro Trp Ala Ala Ser Gly Arg Ala Ile Ala Asn Gly
505                 510                 515

TGC GAC AAG CCG TTT ACC AAG CTG ATT TTT GAT GCC GAA ACC  1596
Cys Asp Lys Pro Phe Thr Lys Leu Ile Phe Asp Ala Glu Thr
    520                 525                 530

GGC CGC ATC ATC GGC GGC GGC ATT GTC GGT CCG AAC GGT GGC  1638
Gly Arg Ile Ile Gly Gly Gly Ile Val Gly Pro Asn Gly Gly
        535                 540                 545

GAT ATG ATC GCG AAG TCT GCC TTG CCA TCG AAA TGG GCT GCG  1680
Asp Met Ile Ala Lys Ser Ala Leu Pro Ser Lys Trp Ala Ala
            550                 555                 560

ACA CGT GCA GAC ATC GGC AAA ACC ATC CAC CCG CGC CCG ACC  1722
Thr Arg Ala Asp Ile Gly Lys Thr Ile His Pro Arg Pro Thr
                565                 570

TTG GGC GAA TCC ATC GGT ATG GCG GCG GAA GTG GCA TTG GGT  1764
Leu Gly Glu Ser Ile Gly Met Ala Ala Glu Val Ala Leu Gly
575                 580                 585

ACT TGT ACC GAC CTG CCT CCG CAA AAG AAA AAA TAA          1800
Thr Cys Thr Asp Leu Pro Pro Gln Lys Lys Lys  *
    590                 595                 599

ATCC GACTGAATAA ACAGCCGATA AGGT TTATTT GA                1836
```

SEQ ID NO: 2

SEQUENCE TYPE : Nucleotide

SEQUENCE LENGTH: 15 bases

ATGCTAGATA AAAGA 15

SEQ ID NO: 3

SEQUENCE TYPE : Amino acid

SEQUENCE LENGTH: 25 amino acids

MOLECULE TYPE: Sequence N-terminal of P64k protein from outer membrane of N. meningitidis

Met Leu Asp Lys Arg Met Ala Leu Val Glu Leu Lys Val Pro Asp
1 5 10 15

Ile Gly Gly His Glu Asn Val Asp Ile Ile
20 25

SEQ ID NO: 4

SEQUENCE TYPE : Nucleotide

SEQUENCE LENGTH: 12 bases

CTAGATAAAA GA 12

SEQ ID NO: 5

SEQUENCE TYPE : Nucleotide

SEQUENCE LENGTH: 8 bases

TCTTTTAT 8

SEQ ID NO: 6

SEQUENCE TYPE : Amino acid

SEQUENCE LENGTH: 221 amino acids
MOLECULE TYPE: Sequence which includes variable regions of P1.15 protein

Leu Gln Leu Thr Glu Pro Pro Ser Lys Ser Gln Pro Gln Val Lys
1 5 10 15

Val Thr Lys Ala Lys Ser Arg Ile Arg Thr Gln Ile Ser Asp Phe
20 25 30

Gly Ser Phe Ile Gly Phe Lys Gly Ser Glu Asp Leu Gly Glu Gly
35 40 45

Leu Lys Ala Val Trp Gln Leu Glu Gln Asp Val Ser Val Ala Gly
50 55 60

Gly Gly Ala Thr Gln Trp Gly Asn Arg Glu Ser Phe Val Gly Leu
65 70 75

Ala Gly Glu Phe Gly Thr Leu Arg Ala Gly Arg Val Ala Asn Gln
80 85 90

Phe Asp Asp Ala Ser Gln Ala Ile Asp Pro Trp Asp Ser Asn Asn
95 100 105

Asp Val Ala Ala Ser Gln Leu Gly Ile Phe Lys Arg His Asp Asp
110 115 120

Met Pro Val Ser Val Arg Tyr Asp Ser Pro Asp Phe Ser Gly Phe
125 130 135

Ser Gly Ser Val Gln Phe Val Pro Ile Gln Asn Ser Lys Ser Ala
140 145 150

Tyr Thr Pro Ala Tyr His Tyr Thr Arg Gln Asn Asn Ala Asp Val
155 160 165

Phe Val Pro Ala Val Val Gly Lys Pro Gly Ser Asp Val Tyr Val
170 175 180

Ala Gly Leu Asn Tyr Lys Asn Gly Gly Phe Ala Gly Ser Tyr Ala
185 190 195

Phe Lys Tyr Ala Arg His Ala Asn Val Gly Arg Asn Ala Phe Glu
200 205 210

Leu Phe Leu Leu Gly Ser Thr Ser Asp Glu Ala
215 220

SEQ ID NO: 7

SEQUENCE TYPE : Amino acid

SEQUENCE LENGTH: 6 amino acids

MOLECULE TYPE: Sequence corresponding to the fusion site between N-terminal of P64k and P1.15

Gly Asp Ala Leu Gln Leu
1 5

SEQ ID NO: 8
SEQUENCE TYPE : Nucleotide
SEQUENCE LENGTH: 19 bases
MOLECULE TYPE: Sequence corresponding to the fusion of N-terminal from gene M-6 and from gene P1.15

GGCGACGCGC TGCAGTTGA 19

SEQ ID NO: 9
SEQUENCE TYPE : Amino acid
SEQUENCE LENGTH: 6 amino acids
MOLECULE TYPE: Sequence corresponding to the fusion site between C-terminal of P64k and P1.15

Glu Ala Asn Ala Tyr Glu
1 5

SEQ ID NO: 10
SEQUENCE TYPE : Nucleotide
SEQUENCE LENGTH: 18 bases
MOLECULE TYPE: Sequence corresponding to the fusion of C-terminal from gene M-6 and from gene P1.15

GAAGCCAACG CGTACGAA 18

SEQ ID NO: 11
SEQUENCE TYPE : Amino acid
SEQUENCE LENGTH: 111 amino acids
MOLECULE TYPE: p64k N-terminal comprising homology region with "lypoil binding site" from E. coli Acetyl transferase

Met Ala Leu Val Glu Leu Lys Val Pro Asp Ile Gly Gly His Glu
1 5 10 15

Asn Val Asp Ile Ile Ala Val Glu Val Asn Val Gly Asp Thr Ile
20 25 30

Ala Val Asp Asp Thr Leu Ile Thr Leu Glu Thr Asp Lys Ala Thr
35 40 45

```
Met Asp Val Pro Ala Glu Val Ala Gly Val Val Lys Glu Val Lys
                  50                  55                  60

Val Lys Val Gly Asp Lys Ile Ser Glu Gly Gly Leu Ile Val Val
                  65                  70                  75

Val Glu Ala Glu Gly Thr Ala Ala Ala Pro Lys Ala Glu Ser Ala
                  80                  85                  90

Ala Ala Pro Arg Lys Lys Pro Leu Lys Cys Arg Trp Val Pro Gln
                  95                 100                 105

Ala Ala Gln Phe Gly Gly
                 110
```

SEQ ID NO: 12

SEQUENCE TYPE : Amino acid

SEQUENCE LENGTH: 112 amino acids

MOLECULE TYPE: "lypoil binding site" from E. coli Acetyl-transferase

```
Val Lys Glu Val Asn Val Pro Asp Ile Gly Gly Asp Glu Val Glu
  1                 5                  10                  15

Val Thr Glu Val Met Val Lys Val Gly Asp Lys Val Ala Ala Glu
                  20                  25                  30

Gln Ser Leu Ile Thr Val Glu Gly Asp Lys Ala Ser Met Glu Val
                  35                  40                  45

Pro Ala Pro Phe Ala Gly Val Val Lys Glu Leu Lys Val Asn Val
                  50                  55                  60

Gly Asp Lys Val Lys Thr Gly Ser Leu Ile Met Ile Phe Glu Val
                  65                  70                  75

Glu Gly Ala Ala Pro Ala Ala Ala Pro Ala Lys Gln Glu Ala Ala
                  80                  85                  90

Ala Pro Ala Pro Ala Ala Lys Ala Glu Ala Pro Ala Ala Ala Pro
                  95                 100                 105

Ala Ala Lys Ala Glu Gly Lys
                 110
```

SEQ ID NO: 13

SEQUENCE TYPE : Amino acid

SEQUENCE LENGTH: 481 amino acids

MOLECULE TYPE: P64k fragment comprising the homology region with Lipoamide Dehydrogenase from E. coli

```
Ser Ala Asp Ala Glu Tyr Asp Val Val Val Leu Gly Gly Gly Pro
  1               5                  10                  15

Gly Gly Tyr Ser Ala Ala Phe Ala Ala Ala Asp Glu Gly Leu Lys
             20                  25                  30

Val Ala Ile Val Glu Arg Tyr Lys Thr Leu Gly Gly Val Cys Leu
         35                  40                  45

Asn Val Gly Cys Ile Pro Ser Lys Ala Leu Leu His Asn Ala Ala
     50                  55                  60

Val Ile Asp Glu Val Arg His Leu Ala Ala Asn Gly Ile Lys Tyr
 65                  70                  75

Pro Glu Pro Ala Leu Asp Ile Asp Met Leu Arg Ala Tyr Lys Asp
             80                  85                  90

Gly Val Val Ser Arg Leu Thr Gly Phe Gly Arg Tyr Gly Glu Lys
         95                 100                 105

Arg Lys Val Asp Val Ile Gln Gly Asp Gly Gln Phe Leu Asp Pro
    110                 115                 120

His His Leu Glu Val Ser Leu Thr Ala Gly Asp Ala Tyr Glu Gln
125                 130                 135

Ala Ala Pro Thr Gly Glu Lys Lys Ile Val Ala Phe Lys Asn Cys
            140                 145                 150

Ile Ile Ala Ala Gly Ser Arg Val Thr Lys Leu Pro Phe Ile Pro
        155                 160                 165

Glu Asp Pro Arg Ile Ile Asp Ser Ser Gly Ala Leu Ala Leu Lys
    170                 175                 180

Glu Val Pro Gly Lys Leu Leu Ile Ile Gly Gly Gly Ile Ile Gly
185                 190                 195

Leu Glu Met Gly Thr Val Tyr Ser Thr Leu Gly Ser Arg Leu Asp
            200                 205                 210

Val Val Glu Met Met Asp Gly Leu Met Gln Gly Ala Asp Arg Asp
        215                 220                 225
```

Leu Val Lys Val Trp Gln Lys Gln Asn Glu Tyr Arg Phe Asp Asn
230 235 240

Ile Met Val Asn Thr Lys Thr Val Ala Val Glu Pro Lys Glu Asp
245 250 255

Gly Val Tyr Val Thr Phe Glu Gly Ala Asn Pro Pro Lys Glu Pro
260 265 270

Gln Arg Tyr Asp Ala Val Leu Val Ala Ala Gly Arg Ala Pro Asn
275 280 285

Gly Lys Leu Ile Ser Ala Glu Lys Ala Gly Val Ala Val Thr Asp
290 295 300

Arg Gly Phe Ile Glu Val Asp Lys Gln Met Arg Thr Asn Val Pro
305 310 315

His Ile Tyr Ala Ile Gly Asp Ile Val Gly Gln Pro Met Leu Ala
320 325 330

His Lys Ala Val His Glu Gly His Val Ala Ala Glu Asn Cys Ala
335 340 345

Gly Thr Lys Ala Tyr Phe Asp Ala Ala Val Ile Pro Gly Val Ala
350 355 360

Tyr Thr Ser Pro Glu Val Ala Trp Val Gly Glu Thr Glu Leu Ser
365 370 375

Ala Lys Arg Pro Ala Gly Lys Ile Thr Lys Ala Asn Phe Pro Trp
380 385 390

Ala Ala Ser Gly Arg Ala Ile Ala Asn Gly Cys Asp Lys Pro Phe
395 400 405

Thr Lys Leu Ile Phe Asp Ala Glu Thr Gly Arg Ile Ile Gly Gly
410 415 420

Gly Ile Val Gly Pro Asn Gly Gly Asp Met Ile Ala Lys Ser Ala
425 430 435

Leu Pro Ser Lys Leu Gly Cys Asp Ala Ala Asp Val Gly Lys Thr
440 445 450

Ile His Pro Arg Pro Thr Leu Gly Glu Ser Ile Gly Met Ala Ala
455 460 465

Glu Val Ala Leu Gly Thr Cys Thr Asp Leu Pro Pro Gln Lys Lys
470 475 480

Lys

SEQ ID NO: 14

SEQUENCE TYPE : Amino acid

SEQUENCE LENGTH: 472 bases

MOLECULE TYPE: Segment of E. coli Lipoamide Dehydrogenase with homology to P64k protein

```
Ser Thr Glu Ile Lys Thr Gln Val Val Val Leu Gly Ala Gly Pro
  1               5                  10                  15

Ala Gly Tyr Ser Ala Ala Phe Arg Cys Ala Asp Leu Gly Leu Glu
             20                  25                  30

Thr Val Ile Val Glu Arg Tyr Asn Thr Leu Gly Gly Val Cys Leu
             35                  40                  45

Asn Val Gly Cys Ile Pro Ser Lys Ala Leu Leu His Val Ala Lys
             50                  55                  60

Val Ile Glu Glu Ala Lys Ala Leu Ala Glu His Gly Ile Val Phe
             65                  70                  75

Gly Glu Pro Lys Thr Asp Ile Asp Lys Ile Thr Trp Lys Glu Lys
             80                  85                  90

Val Ile Asn Gln Leu Thr Gly Gly Leu Ala Gly Met Ala Lys Gly
             95                 100                 105

Arg Lys Val Lys Val Val Asn Gly Leu Gly Lys Phe Thr Gly Ala
            110                 115                 120

Asn Thr Leu Glu Val Glu Gly Glu Asn Gly Lys Thr Val Ile Asn
            125                 130                 135

Phe Asp Asn Ala Ile Ile Ala Ala Gly Ser Arg Pro Ile Gln Leu
            140                 145                 150

Pro Phe Ile Pro His Glu Asp Pro Arg Ile Trp Asp Ser Thr Asp
            155                 160                 165

Ala Leu Glu Leu Lys Glu Val Pro Glu Arg Leu Leu Val Met Gly
            170                 175                 180

Gly Gly Ile Ile Gly Leu Glu Met Gly Thr Val Tyr His Ala Leu
            185                 190                 195

Gly Ser Gln Ile Asp Val Val Glu Met Phe Asp Gln Val Ile Pro
            200                 205                 210

Ala Ala Asp Lys Asp Ile Val Lys Val Phe Thr Lys Arg Ile Ser
            215                 220                 225
```

```
Lys Lys Phe Asn Leu Met Leu Glu Thr Lys Val Thr Ala Val Glu
                230                 235                 240

Ala Lys Glu Asp Gly Ile Tyr Val Thr Met Glu Gly Lys Lys Ala
                245                 250                 255

Pro Ala Glu Pro Gln Arg Tyr Asp Ala Val Leu Val Ala Ile Gly
                260                 265                 270

Arg Val Pro Asn Gly Lys Asn Leu Asp Ala Gly Lys Ala Gly Val
                275                 280                 285

Glu Val Asp Asp Arg Gly Phe Ile Arg Val Asp Lys Gln Leu Arg
                290                 295                 300

Thr Asn Val Pro His Ile Phe Ala Ile Gly Asp Ile Val Gly Gln
                305                 310                 315

Pro Met Leu Ala His Lys Gly Val His Glu Gly His Val Ala Ala
                320                 325                 330

Glu Val Ile Ala Gly Lys Lys His Tyr Phe Asp Pro Lys Val Ile
                335                 340                 345

Pro Ser Ile Ala Tyr Thr Glu Pro Glu Val Ala Trp Val Gly Leu
                350                 355                 360

Thr Glu Lys Glu Ala Lys Glu Lys Gly Ile Ser Tyr Glu Thr Ala
                365                 370                 375

Thr Phe Pro Trp Ala Ala Ser Gly Arg Ala Ile Ala Ser Asp Cys
                380                 385                 390

Ala Asp Gly Met Thr Lys Leu Ile Phe Asp Lys Glu Ser His Arg
                395                 400                 405

Val Ile Gly Gly Ala Ile Val Gly Thr Asn Gly Gly Glu Leu Leu
                410                 415                 420

Gly Glu Ile Gly Leu Ala Ile Glu Met Gly Cys Asp Ala Glu Asp
                425                 430                 435

Ile Ala Leu Thr Ile His Ala His Pro Thr Leu His Glu Ser Val
                440                 445                 450

Gly Leu Ala Ala Glu Val Phe Glu Gly Ser Ile Thr Asp Leu Pro
                455                 460                 465

Asn Pro Lys Ala Lys Lys Lys
                470
```

## Claims

1. A recombinant polynucleotide, comprising a nucleotide sequence coding for a protein P64k of Neisseria

meningitidis, said protein P64k essentially having the amino acid sequence shown in SEQ ID NO:1.

2. A recombinant polynucleotide according to claim 1, wherein said nucleotide sequence coding for the protein P64k of N. meningitidis essentially consists of the nucleotide sequence shown in SEQ ID NO:1.

3. A recombinant polynucleotide according to claim 1 or 2, further comprising a nucleotide sequence of a cloning or expression vector.

4. A transformed microorganism, containing a recombinant polynucleotide according to any of claims 1 to 3.

5. A transformed microorganism according to claim 4, which is capable of expressing the protein P64k of N. meningitidis.

6. A transformed microorganism according to claim 5, which is an Escherichia coli strain, e.g. E. coli strain HB101, transformed with an expression vector containing a nucleotide sequence coding for the protein P64k of N. meningitidis, e.g. the expression vector pM-6.

7. A recombinant proteinaceous substance, comprising an amino acid sequence corresponding to the amino acid sequence of at least a part of a protein P64k of N. meningitidis, said protein P64k essentially having the amino acid sequence shown in SEQ ID NO:1.

8. A recombinant proteinaceous substance according to claim 7, which is a fusion protein or a protein/polysaccharide conjugate comprising the amino acid sequence of protein P64k of N. meningitidis.

9. A vaccine composition, comprising a recombinant protein according to claim 7 or 8, together with a suitable carrier, diluent or adjuvant.

10. A vaccine composition, comprising a lipoamide dehydrogenase or acetyl transferase capable of inducing antibodies which can bind a protein P64k of N. meningitidis, together with a suitable carrier, diluent or adjuvant.

11. Monoclonal antibody, raised against a recombinant proteinaceous substance according to claim 7 or 8, or against a lipoamide dehydrogenase or acetyl transferase, and capable of binding a protein P64k of N. meningitidis.

12. A process for preparing a protein P64k of Neisseria meningitidis, or a fusion protein comprising protein P64k, said protein P64k essentially having the amino acid sequence shown in SEQ ID NO:1, comprising the steps of transforming a microorganism with an expression vector containing a nucleotide sequence coding for said protein P64k, or said fusion protein, culturing the transformed microorganism to obtain expression of said protein P64k, or said fusion protein, and isolating said expression product.

13. A method for the isolation and expression of a gene which codes for a protein belonging to the outer membrane of N. meningitidis and its use in vaccine preparations, wherein the gene from the strain of N. meningitidis B:4:P1.15, identified as M-6, is obtained from the screening of a genomic library in the EMBL 3, and is cloned and expressed in a suitable host, coding for a protein of 64kDa belonging to the outer membrane of N. meningitidis which has bactericidal activity against different serogroups, serotypes and subtypes of N. meningitidis.

14. A nucleotide sequence obtained by the method of claim 13, characterized in that the sequence is corresponding with the M-6 gene and codes for the P64k protein, identified in the sequence listing with number 1.

15. A recombinant DNA according to claims 13 and 14, characterized in that it is a molecule which contains the M-6 gene which codes for P64k protein.

16. A recombinant DNA according to claim 15, characterized in that it is a phage, a plasmid or an expression vector.

17. A recombinant DNA according to claims 15 and 16, characterized in that it is the phage EMBL 3, plasmid pM-1 and expression vector pM-6.

18. A transformed microorganism according to the preceding claims, characterized in that it results from the transformation of a suitable host, carries the M-6 gene, expresses high levels of P64k protein and maintains good viability and cellular stability.

19. A transformed microorganism according to claim 18, characterized in that it is the clone HBM64 which is obtained from the transformation of E. coli host strain HB101 with the expression vector pM-6, and it presents high stability and viability and expression levels of P64k protein higher than 25 % of the total protein of the cell.

20. A recombinant protein obtained according to the preceding claims, characterized in that it essentially has the amino acid sequence shown in the sequence listing under SEQ ID NO:1.

21. Protein P64k according to preceding claims characterized in that it is the direct result of the expression of the M-6 gene as well as any peptide which has important immunological regions obtained by chemical synthesis or enzymatic degradation of P64k protein and its use in fusion proteins and polysaccharide protein conjugates for monovalent and multivalent vaccine preparations.

22. A vaccine preparation comprising protein P64k and at least one carrier, diluent or adjuvant used for vaccine preparations, as well as comprising any protein of Lipoamide dehydrogenase or Acetyl transferase from other organism or modification thereof, which is able to induce antibodies against the P64k protein.

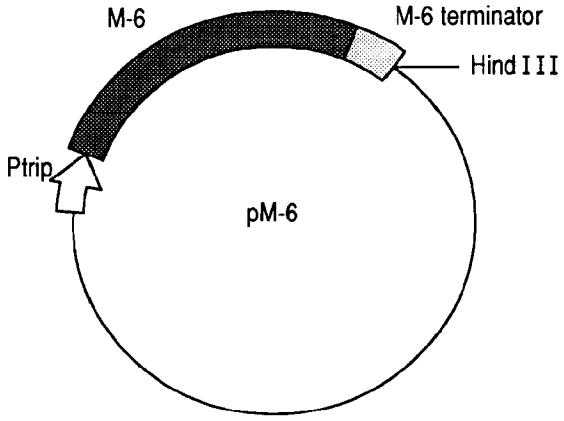

M-6
M-6 terminator
Hind III
Ptrip
pM-6

FIG. 1

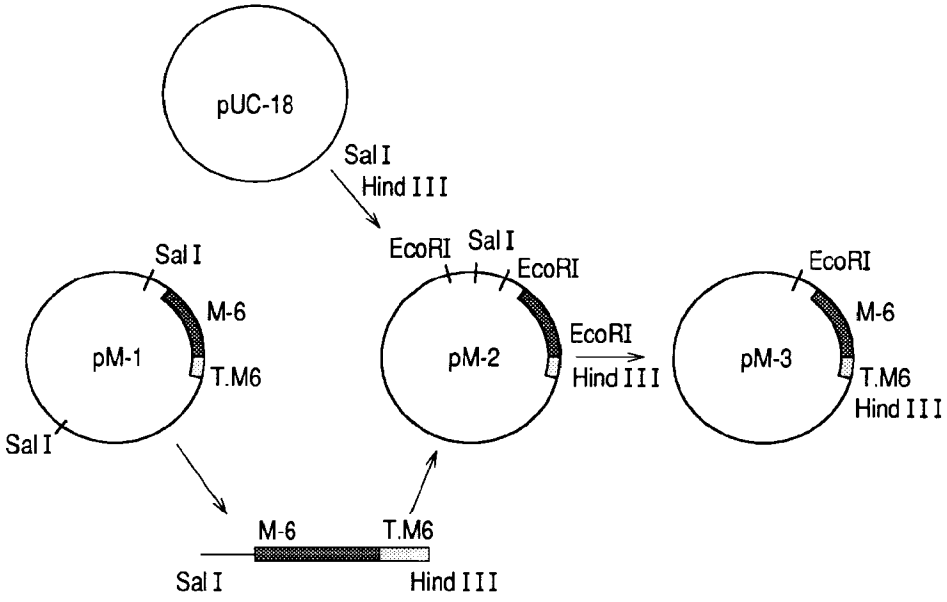
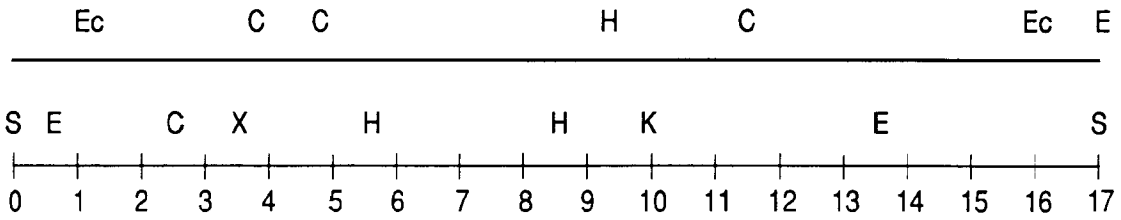

pUC-18
Sal I
Hind III
Sal I
M-6
pM-1
T.M6
Sal I
EcoRI
Sal I
EcoRI
pM-2
EcoRI
Hind III
EcoRI
M-6
pM-3
T.M6
Hind III
M-6
T.M6
Sal I
Hind III

FIG. 2




S. Sal I ; E. EcoRV ; Ec. EcoR I ;C. Cla I ;
X. Xho I ; H. Hind III ; K. Kpn I .

FIG. 3

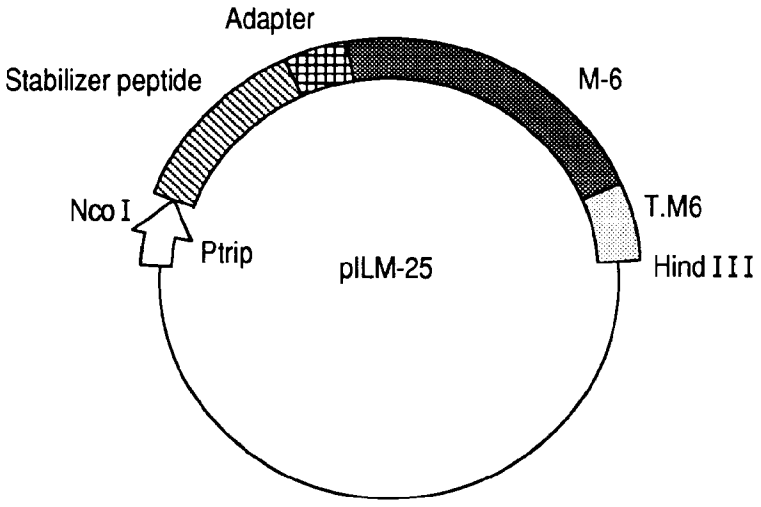



FIG. 4

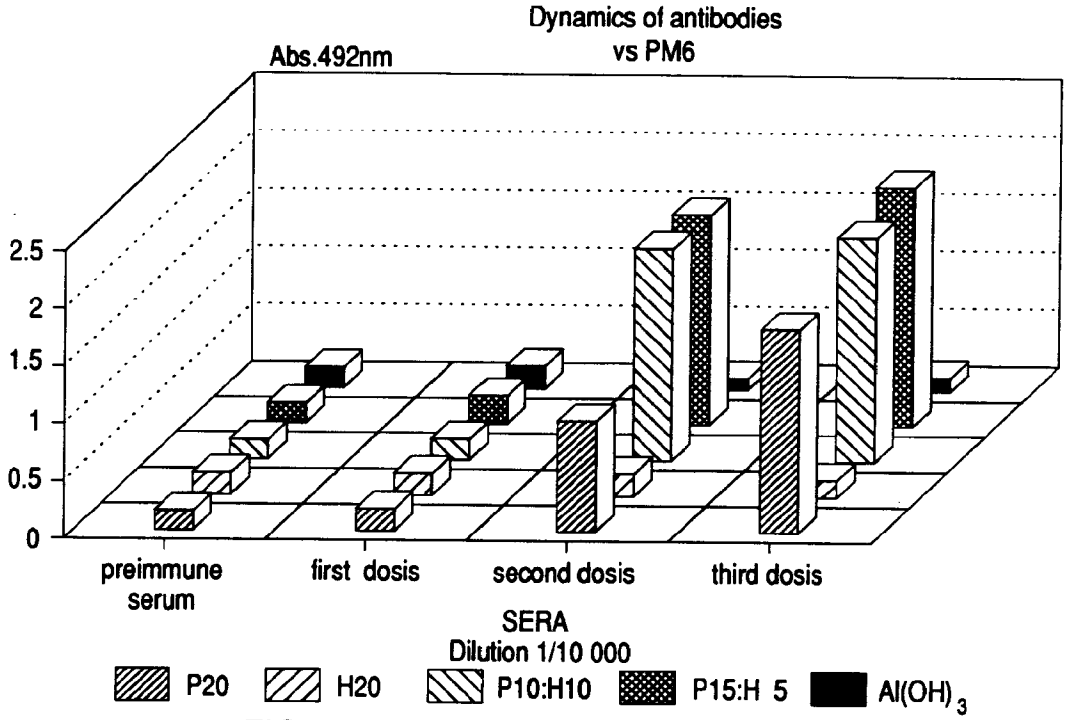

Dynamics of antibodies
vs PM6
Abs.492nm
2.5
2
1.5
1
0.5
0
preimmune serum
first dosis
second dosis
third dosis
SERA
Dilution 1/10 000
P20
H20
P10:H10
P15:H 5
Al(OH)3

FIG. 5

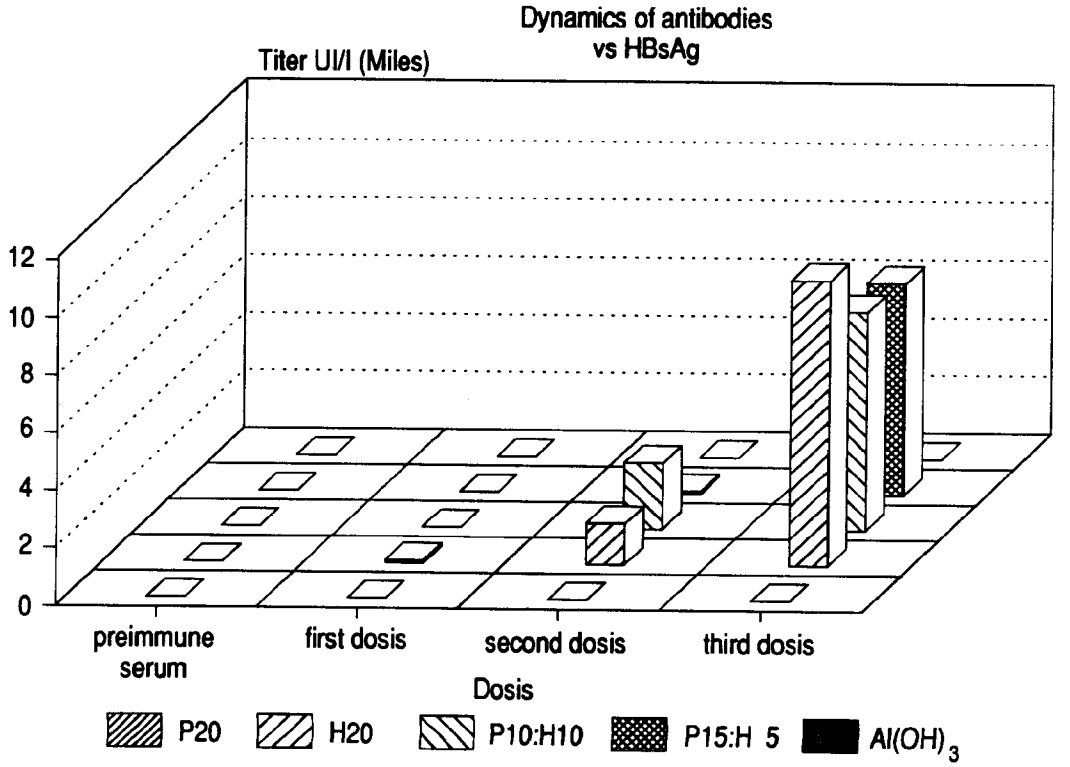

Dynamics of antibodies
vs HBsAg
Titer UI/l (Miles)
12
10
8
6
4
2
0
preimmune serum
first dosis
second dosis
third dosis
Dosis
P20
H20
P10:H10
P15:H 5
Al(OH)3

FIG. 6